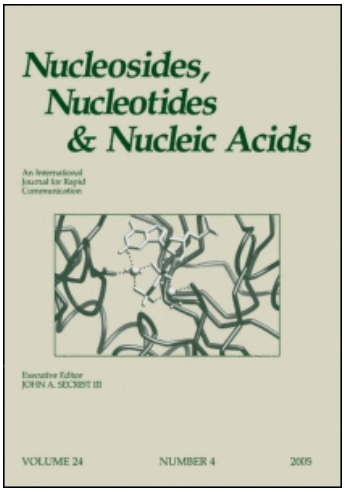

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF A BIS-SUBSTITUTED 3'-DEOXYADENOSINE ANALOG OF 2-5A

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Abstract - The 5'-monophosphate, p5'(3'dA)2'p5'A2'5'(3'dA), was synthesized and found to bind to the 2-5A-dependent endonuclease of mouse L cells only two-three times less effectively than the parent p5'A2'p5'A2'p5'A. When evaluated for its ability to activate the 2-5A-dependent endonuclease, ppp5'(3'dA)2'p5'A2'p5'(3'dA) was found to be fifty times more effective than ppp5'A2'p5'(3'dA)2'p5'A and ten times less effective than 2-5A as an endonuclease activator.

The interaction of 2-5A [5'-O-triphosphoryladenylyl(2' 5')adenylyl (2' 5')adenosine] with its target enzyme, RNase L or the 2-5A-dependent endonuclease,[1] has been found thus far to depend on several key features of the 2-5A molecule.[2] These strategic domains of 2-5A include the 5'-phosphate moiety, the heterocyclic bases, and the ribose-phosphate backbone. Most recently, we have demonstrated that of the three 3'-hydroxyl moieties of the 2-5A trimer molecule, the first and third can be converted to hydrogen without any substantial adverse effect on resultant binding to or activation of RNase L[3]. Only the second 3'-hydroxyl group was critical for activation of RNase L.

Because these findings are to be used to design 2-5A analogs which per se may possess RNase L-activating activity in the intact cell, it

was important to provide an independent confirmation. According to the above findings, if the first and third 3'-hydroxyl groups of 2-5A are not vital for RNase L binding and activation, then the bis-substituted analog, ppp5'(3'dA)2'p5'A2'p5'(3'dA), should possess an RNase L activating ability similar to 2-5A itself. In this paper, we describe the synthesis and biological evaluation of such a molecule.

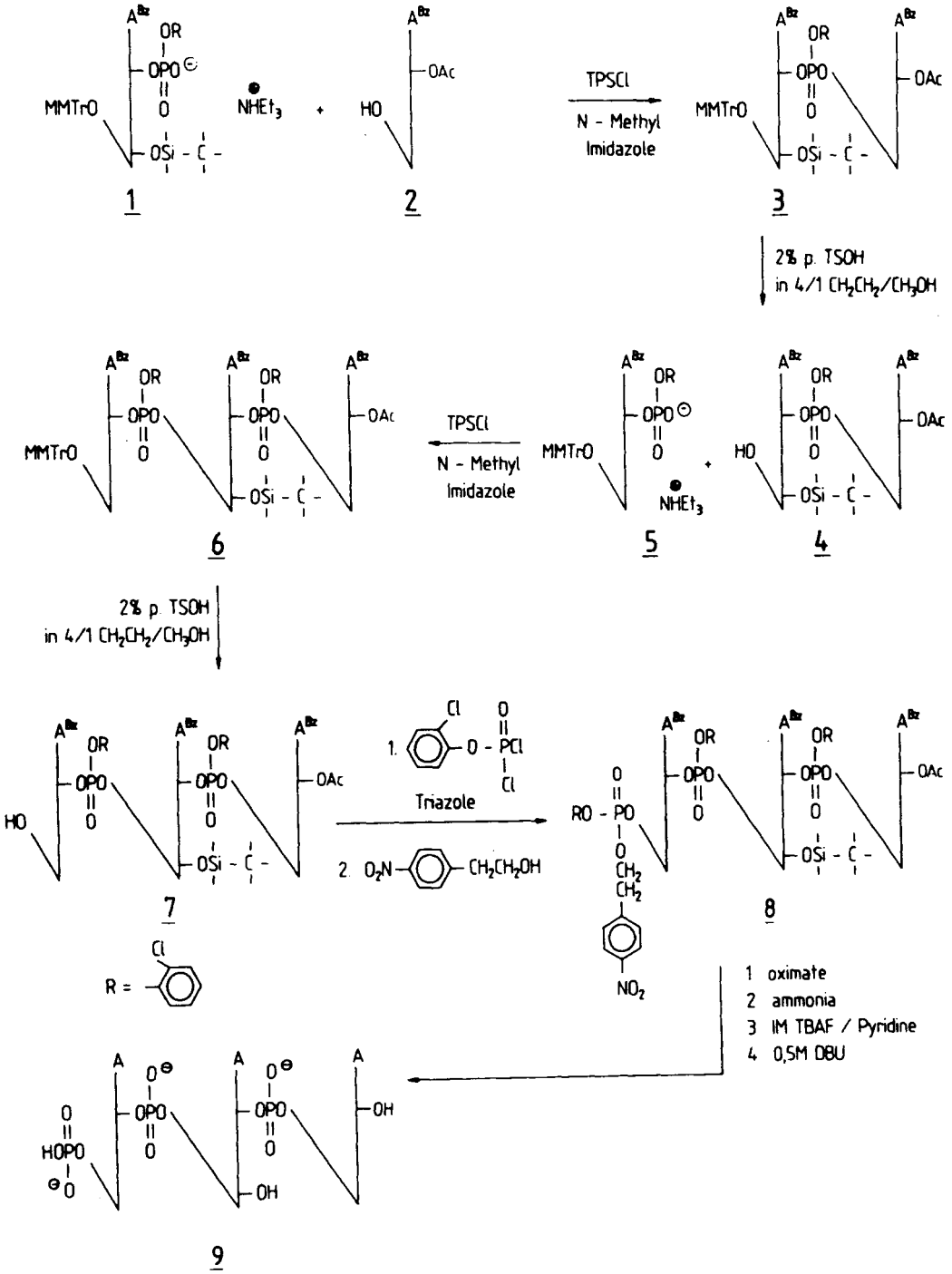
RESULTS

CHEMISTRY (Scheme 1)

To prepare the oligonucleotide 5'-monophosphate, p5'(3'dA)2'p5'A2'p5'(3dA), the phosphodiester 1[4] was condensed with N^6-benzoyl-2'-O-acetyl-3'-deoxyadenosine[5] (2) using triisopropylbenzenesulfonyl chloride and N-methylimidazole. The resulting product (3) was treated with 2% p-toluenesulfonic acid to remove the monomethoxytrityl group and to give 4 which was condensed with the product of the reaction of N^6-benzoyl-5'-O-monomethoxytrityl-3'-deoxyadenosine[6] (5) and o-chlorophenylphosphorodichloridate. The product, 6, was de-monomethoxytritylated with 2% p-toluenesulfonic acid to yield 7. By phosphorylation with o-chlorophenyl phosphorodichloridate and reaction with p-nitrophenylethanol, 7 was converted to the protected 5'-monophosphate 8. Sequential treatment of 8 with p-nitrobenzaldoxime and tetramethylguanidine and ammonia, tetrabutylammonium fluoride and diazabicyclo[5.4.0]undecene (DBU) provided the requisite product 9.

The 5'-triphosphate, ppp5'(3'dA)2'p5'A2'p5'(3'dA) (10), was generated in the usual manner by first converting the 5'-monophosphate to the 5'-phosphoroimidazolidate by a modification of the procedure of Mukaiyama and Hashimoto.[7] The imidazolidate was isolated as the sodium salt, and this was reacted with tri-n-butylammonium pyrophosphate to give, after purification, the desired 5'-triphosphate 10.

Corroboration of the assigned structure for compound 9, the 5'-monophoshate, was obtained through several different approaches. The proton NMR of 9 displayed 6 aromatic protons corresponding to the adenosine ring protons while three anomeric protons could be located in



SCHEME 1

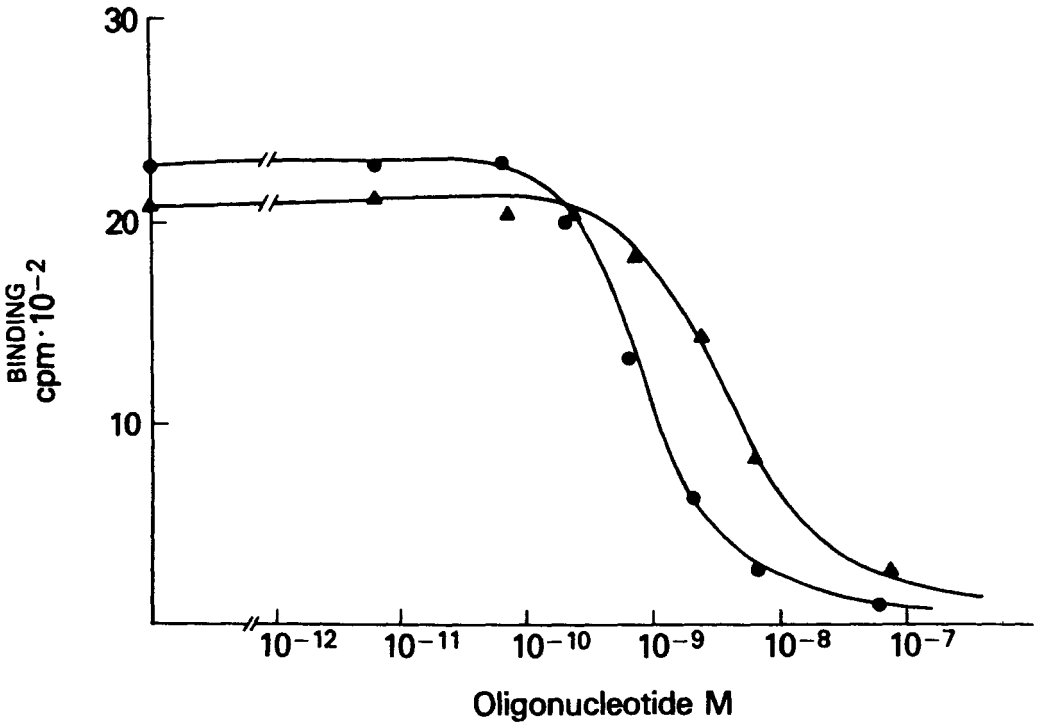


FIG. 1a. Ability of p5'(3'dA)2'p5'A2'p5'(3'dA) to inhibit the binding of ppp5'A2'p5'A2'p5'A2'p5'A3'[^{32}P]pCp to the 2-5A-dependent endonuclease of mouse L cell extracts. ▲, p5'(3'dA)2'p5'A2'p5'(3'dA); ●, ppp5'A2'p5'A2'p5' as control.

the usual range at 5.6-5.9 ppm. Alkaline degradation of 9 with 0.5 M KOH gave two products, 3'-deoxyadenosine and a dinucleotide diphosphate, tenatively identified as p5'(3'dA)2'p5'A2'[3']p, in the appropriate (1:1) stoichiometric ratio. Bacterial alkaline phosphatase digestion of 9 gave the "core" oligomer, (3'dA)2'p5'A2'p5'(3'dA). Finally, when 9 was digested with snake venom phosphodiesterase, 3'-deoxyadenosine 5'-monophosphate and adenosine 5'-monophosphate were obtained in the predicted stoichiometric ratio of 2:1, respectively.

Enzymic and chemical degradations also were employed to corroborate the structure of the triphosphate 10. Thus bacterial alkaline phosphatase digestion of 10 gave the original "core" trimer, (3'dA)2'p5'A2'p5'(3'dA). Digestion with KOH yielded 3'-deoxyadenosine and the same dinucleotide diphosphate as obtained in the alkaline

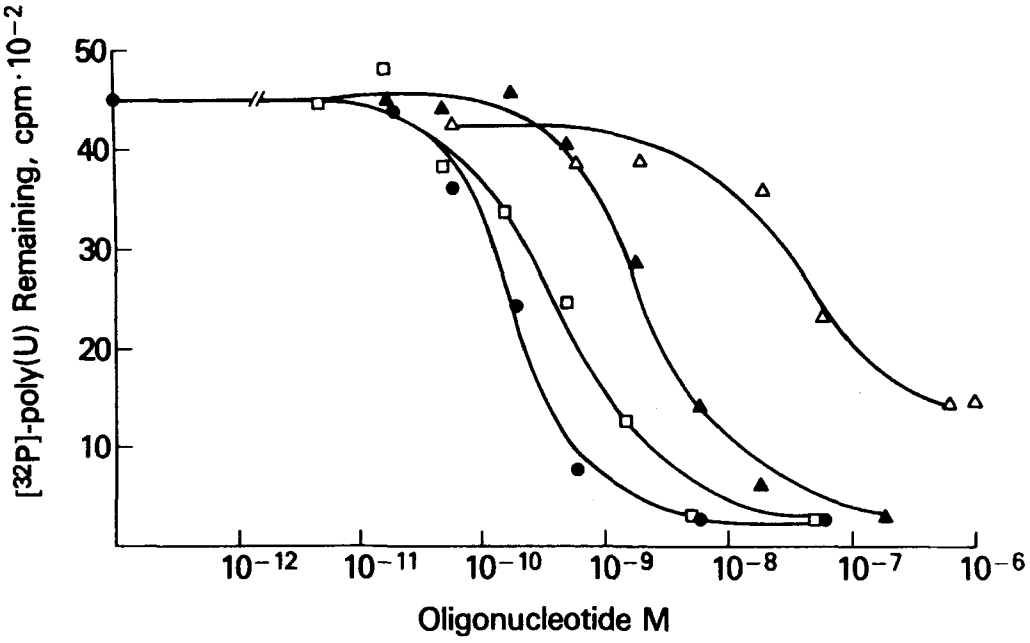


FIG. 1b. Core cellulose assay for the activation of the 2-5A-dependent endonuclease (RNase L) by various analogs of 2-5A. The assay was performed according to the procedures previously described.[3,9,11] Activation of the nuclease was determined by conversion of poly(U)-3'-[^{32}P]pCp to acid-soluble fragments after incubation with the enzyme immobilized on core cellulose. The legend is: △, ppp5'A2'p5'(3'dA)2'p5' A; ▲, ppp5'(3'dA)2'p5'A2'p5'(3'dA); □, ppp(3'dA)2'p5'A2'p5'A; ●, ppp5'A2'p5'A2'p5'A, control.

digestion of the 5'-monophosphate 9. As in the previous example, the stoichiometric ratio of products was approximately 1:1. Snake venom phosphodiesterase produced from 10 two products: 3'deoxyadenosine 5'-monophosphate and 5'-AMP in the predicted ratio of 2:1.

BIOCHEMICAL EVALUATION

I. Binding to Target Enzyme RNase L. This was determined in the usual manner by ascertaining the ability of the analog, p5'(3'dA)2'p5'A2'p5'(3'dA), to prevent the binding of a radiolabeled probe to the endonuclease.[8] From the data of Figure 1a, it can be seen that the 5'-monophosphate was bound to RNase L approximately 5 times

less effectively than the 5'-triphosphate, 2-5A itself. Since the parent 5'-monophosphate, p5'A2'p5'A2'p5'A, was bound to RNase L 2-3 times less effectively than 2-5A (data not illustrated), this indicated that the affinity of the bis-cordycepin-substituted analog, 9, was only two-three times less than its parent, p5'A2'p5'A2'p5'A.

II. Activation of the Target Enzyme, RNase L. The ability of ppp5'(3'dA)2'p5'A2'p5'(3'dA) to activate RNase L was determined using a functional assay developed by Silverman.[9] This assay involved immobilization and partial purification of RNase L from mouse L cell extracts on "core"cellulose (A2'p5'A2'p5'A2'p5'A-cellulose). The activation of RNase L by 2-5A or analog was monitored by the degradation of poly(U)-3'-[^{32}P]pCp. The results are shown in Figure 1b. In these experiments, ppp5'(3'dA)2'p5'A2'p5'(3'dA) was compared to 2-5A itself (ppp5'A2'5'A2'p5'A) as well as to ppp5'A2'p5'(3'dA)2'p5'A and ppp5'(3'dA)2'p5'A2'p5'A. Under these conditions, the most effective activator of RNase L was 2-5A itself (EC_{50} or concentration to effect 50% degradation of labeled probe = 2×10^{-10} M). The analog bearing a 3'-deoxyadenosine residue at the 5'-position only (ppp5'(3'dA)2'p5'A2'-p5'A) was slightly less effective (EC_{50}= 5×10^{-10} M). The bis-substituted analog was about 10 times less active then 2-5A since its EC_{50} was 2×10^{-9} M. Finally by far the least active analog was ppp5'A2'p5'(3'dA)-2'p5'A with the second or central nucleotide unit of 2-5A substituted by 3'-deoxyadenosine. Its EC_{50} was 5×10^{-8} M. In addition, its intrinsic activity was lower than the other analogs since even at concentrations well above its EC_{50}, it failed to cause degradation of all the substrate. These results with the mono-substituted cordycepin analogs are in good agreement with the earlier observations.[3]

Discussion and Conclusions

Earlier work[3] suggested that since only the 3'-hydroxyl group of the second adenosine nucleotide unit of 2-5A was required for RNase L activation, the 3'-hydroxyls of both the 5'-terminal and 2'-terminal adenosines of 2-5A might be simultaneously replaceable without substantially decreasing RNase L-activating properties of the oligonucleotide. In part, this suppositon seems corroborated by the present work. Thus, ppp5'(3'dA)2'p5'A2'p5'(3'dA) possessed 50 times the

RNase L activating ability of ppp5'A2'p5'(3'dA)2'p5'A. In addition, its intrinsic activity, as measured by its ability to effect apparent complete degradation of RNA probe, was greater than that of ppp5'A2'p5'(3'dA)2'p5'A (Figure 1b). On the other hand, however, ppp5'(3'dA)2'p5'A2'p5'(3'dA) possessed only one tenth the RNase L activation ability of 2-5A and only 1/5 the activation ability of ppp5'(3'dA)2'p5'A2'p5'A. Moreover, ppp5'(3'dA)2'p5'A2'p'5(3'dA) would have only one-fiftieth (1/50) of the activation ability of the 2'-terminally-substituted analog, ppp5'A2'p5'A2'p5'(3'dA) previously shown[3] to be approximately 5 times more active that 2-5A. Apparently, bis-1,3-substitution of the 2-5A molecule by 3'-deoxyadenosine does not result in a biological activity that can be predicted by simply summing the effect of each individual mono-substitution of the 2-5A oligomer. Whether or not conformational constraints may govern the biological activty of such a bis-substituted 2-5A is a subject for future investigation.

Even in the absence of such considerations, it is apparent that ppp5'(3'dA)2'p5'A2'p5'(3'dA) is a molecule of considerable biological potency. As such it proves a useful simplified model for the future synthesis of potential RNase L activators, especially those which may have the capacity to enter an intact cell.

EXPERIMENTAL

Sources of chemical and isotopes as well as assay procedure have been extensively described elsewhere[3-6,9-11]. HPLC chromatography was carried out using the indicated columns and solvents with a Beckman system with two model 110A pumps and a model 421 controller. Proton NMR spectra were recorded on a Bruker WM250 spectrometer and chemical shifts are in δ (ppm).

Preparation of p5'(3'dA)2'p5'A2'p5'(3'dA)

N^6-Benzoyl-3'-O-tert.butyldimethylsilyl-5'-O-monomethoxytrityladenylyl-[2'-[O^P-(o-chlorophenyl)]-5']-N^6-benzoyl-2'-O-acetyl-3'-deoxyadenosine (3).

The phosphodiester 1[4] (750 mg, 0.75 mmol) and N^6-benzoyl-2'-O-acetyl-3'-deoxyadenosine[5] (2) (278 mg, 0.7 mmol) were coevaporated with

dry pyridine (3x3 ml) and dissolved in dry pyridine (7 ml). Triisopropylbenzenesulfonylchloride (462 mg, 1.5 mmol) and N-methylimidazole (0.25 ml, 4.5 mmol) were added and the reaction stirred for 2 h at 25°C. The reaction mixture was extracted with chloroform (200 ml), washed with water (2x100 ml), dried over Na_2SO_4 and evaporated. The residue was purified by silica gel column chromatography (2.5x20 cm) and eluted with $CHCl_3$ then $CHCl_3/CH_3OH$ (99/1). The product fractions were collected and evaporated to give a solid foam (747 mg, 79%). UV λ max 279 nm (log ϵ 4.59) and 231 nm (log ϵ = 4.61). NMR($CDCl_3$): 8.69 (1H, S), 8.60 (1H, s), 8.18 (1H, s), 8.00 (1H, s) all purine 2 and 8 H's; 6.35 (1H, d) and 6.25 (1H, d) anomeric protons; 3.73 (3H, s, OCH_3); 2.09 and 2.07 (1H, S, acetate CH_3 of diastereomers); 0.82 (s), 0.80 (s), $(CH_3)_3C$.; 0.037 (s), 0.075 (s), -0.0027 (s), -0.024(s), $(CH_3)_2Si$=. Anal. Calcd for $C_{68}H_{68}N_{10}O_{13}SiClP.\ H_2O$: C, 60.68; H, 5.24; N, 10.40. Found: C, 60.02; H, 5.36; N, 10.64.

N^6-Benzoyl-3'-O-tert.butyldimethylsilyladenylyl-[2'-[O^P-(o-chlorophenyl)]-5']- N^6-benzoyl-2'-O-acetyl-3'-deoxyadenosine (4).

Compound 3 (630 mg, 0.5 mmol) was stirred with 2% p-toluenesulfonic acid in CH_2Cl_2/CH_3OH (4/1) (10 ml) for 45 min. Phosphate buffer (pH 7.0) (15 ml) was added and the mixture was extracted with $CHCl_3$ (100 ml). The $CHCl_3$-phase was washed with water (2x50 ml), evaporated to dryness and the substance was purified by silica gel chromatography (2x15 cm) and subsequent elution with $CHCl_3$ then $CHCl_3/CH_3OH$ (100/1) to give a solid foam (450 mg, 90%).

Triethylammonium-N^6-benzoyl-5'-O-monomethoxytrityl-3'-deoxyadenosine-2'-(-o-chlorophenyl)phosphate (5).

To an ice-cold solution of 1,2,4-triazole (280 mg, 4 mmol) and o-chlorophenylphorosphodichloridate (470 mg, 1.9 mmol) in pyridine (6 ml) was added N^6-benzoyl-5'-O-monomethoxytrityl-3'-deoxyadenosine[6] (877 mg, 1.4 mmol) in pyridine (6 ml) dropwise. After 30 min the reaction mixture flask was cooled in an ice-bath. 1.5 ml 90% aqueous pyridine was added and after 10 min stirring, the residue was extracted with chloroform (100 ml), washed with water (2x50 ml), dried and evaporated,

and finally coevaporated with pyridine (3x10 ml), to give 5, used as such for condensation.

N^6-Benzoyl-5'-O-monomethoxytrityl-3'-deoxyadenylyl-[2'-[O^P-(o-chlorophenyl)]-5']- N^6-benzoyl-3'-O-tert.butyldimethylsilyl-adenylyl-[2'-[O^P-(o-chlorophenyl)]-5']- N^6-benzoyl-2'-O-acetyl-3'-deoxyadenosine (6).

The phosphodiester 5 (1.4 mmol) and 5'-hydroxy dimer 4 (1.05 g, 1 mmol) were coevaporated with pyridine (3x3 ml) and dissolved in dry pyridine (10 ml). Triisopropylbenzenesulfonylchloride (862 mg, 2.8 mmol) and N-methylimidazole (0.46 ml, 8.4 mmol) were added and the mixture stirred for 2 h at 23°C. The reaction mixture was extracted with chloroform (200 ml) and washed with water (2x100 ml), dried over Na_2SO_4 and evaporated. The residue was purified by silica gel chromatography (2.5x15 cm) and eluted with $CHCl_3$ and $CHCl_3/CH_3OH$ (100/1). The product fractions were collected and evaporated to give a solid foam (1.65 g, 89%). UV λ max 271 nm (log ε 4.71). NMR ($CDCl_3$): 8.66 (3H, m) and 8.18 (3H, m) purine 2 and 8 protons; 6.23 (1H, m), 6.19 (1H, m) and 6.03 (1H, m), all anomeric protons; 3.72 (3H, s, OCH_3); 2.07 and 2.03 (s, CH_3 of acetyl, diastereomers); 0.876 (s), 0.870 (s) and 0.866 (s), $(CH_3)_3$ C-; 0.027 (m), 0.072 (m), 0.077 (m), 0.085 (m) and 0.095(m), $(CH_3)_2$ Si=. Anal. Calcd for $C_{91}H_{87}N_{15}O_{19}P_2SiCl_2.H_2O$: C, 57.69; H, 4.78; N, 11.21. Found: C, 57.35; H, 4.72; N, 10.83.

N^6-Benzoyl-3'-deoxyadenylyl-[2'-[O^P-(o-chlorophenyl)]-5']-N^6-benzoyl-3'--O-tert. butyldimethylsilyl-adenylyl-[2'-[O^P-(o-chlorophenyl)]-5']-N^6-benzoyl- 2'-O-acetyl- 3'-deoxyadenosine (7).

Compound 6 (100 mg, 0.55 mmol) was stirred with 2 % p-toluenesulfonic acid in CH_2Cl_2/CH_3OH (4/1) (2 ml) for 30 min. Phosphate buffer (pH 7.0) (5 ml) was added and the reaction mixture was extracted with $CHCl_3$ (50 ml). The $CHCl_3$-phase was washed with water (2x25 ml), evaporated and the substance was purified by silica gel chromatography (2.5x10cm) with elution with $CHCl_3$ and then $CHCl_3/CH_3OH$ (98/2) followed by $CHCl_3/CH_3OH$ (95/5) to give a solid foam (80 mg, 93%).

N^6-Benzoyl-5'-O-(o-chlorophenyl, p-nitrophenylethyl)-phosphoryl-3'-deoxyadenylyl-[2'-[O^P-(o-chlorophenyl)]-5']-N^6-benzoyl-3'-O-tert.-butyl-

dimethylsilyl-adenylyl-[2'-[O^P-(o-chlorophenyl)]-5']-N^6-benzoyl-2'-O-acetyl- 3'-deoxyadenosine (8).

To a solution of 1,2,4-triazole (11.4 mg, 0.083 mmol) and o-chlorophenylphosphorodichloridate (22 mg, 0.077 mmol) in dry pyridine (0.3 ml) was added the 5'-hydroxy trimer 7 (80 mg, 0.049 mmol). After 0.5 h stirring, p-nitrophenylethanol (40 mg, 0.24 mmol) was added and the reaction mixture was kept at room temp. for 16 h. The solution was extracted with $CHCl_3$ (3x30 ml), washed with water (2x15 ml), evaporated to dryness and the residue coevaporated with toluene (2x10 ml). Purificaton was done on a preparative thin layer chromatography plate (20x20x0.2 cm) with $CHCl_3/CH_3OH$ (95/5) to give a solid foam (89 mg, 93 %).

5'-O-Phosphoryl-3'-deoxyadenylyl-(2'→5')-adenylyl-(2'→5')-3'-deoxyadenosine (9).

p-Nitrobenzaldoxime (183 mg, 1.09 mmol) and tetramethylguanidine (177 mg, 1.53 mmol) were stirred in dioxane/water (1/1) (5 ml) and added to the compound 8 (50 mg, 25 µl). After 16 h at room temp., the solution was evaporated to dryness and the residue was treated with 25 % NH_4OH (20 ml). After 48 h stirring, the reaction mixture was evaporated to dryness, taken up in water (50 ml), and washed with $CHCl_3$ (2x25 ml). Thin layer chromatography showed the presence of the required product in water phase, so it was evaporated to dryness and finally coevaporated with pyridine (4x4 ml) and THF (1x4 ml) and the residue was treated with 1 M tetrabutylammonium fluoride in pyridine (5 ml) for 48 h. After evaporation and coevaporation with dry pyridine (4x4ml), 0.5 M DBU in dry pyridine (7.5 ml) was added. After 24 h stirring at 23°C the solution was evaporated to dryness, the residue taken up in water (50 ml) and washed with $CHCl_3$ (4x20 ml). The water phase was concentrated to a small volume which was put on a DEAE Sephadex A-25 column (1x60 cm). Separation employed a linear gradient of 0.001-0.7 M triethylammonium bicarbonate (pH 7.0). The main fractions were collected and evaporated to dryness and coevaporated several times with water to remove triethylamine. Finally they were purified by paper chromatography in the system n-PrOH/NH_4OH/water (55/10/35) to give 9 in form of its ammonium salt (709 O.D. units, 78%) based on ε = 36000 (neglecting possible hypochromicity).

Compound 9 had an HPLC retention time of 2.86 min and was homogeneous on a Lichrosorb RP18 column (2.5 mm x 4.6 cm) using isocratic elation with 0.1 m ammonium acetate/acetonitrile (95/5) at 1 ml/mm. Degradation with 0.5 N KOH gave p5'(3'dA)2'p5'A(2')3'p and 3'dA in a ratio of 1.00 to 1.09. Snake venom phosphodresterase digestion yielded p5'(3'dA) and 5'AMP in a ratio of 2.11:1.00, respectively. Bacterial alkaline phosphatase digestion gave but one product: putative "core" (3'dA)2'p5'A2'p5'(3'dA). NMR (D_2O): 8.03 (3H, s), 7.81 (1H, s), 7.78 (2H, s) all purine 2 and 8 protons; 5.87 (1H, apparent s, $J \leq 1$), 5.80 (1H, d, J=2.5 Hz), 5.63 (1H, apparent s, $J \leq 1$), all anomeric protons.

Preparation of ppp5'(3'dA)2'p5'A2'p5'(3'dA) (10)

The triethylammonium salt of p5'(3'dA)2'p5'A2'p5'(3'dA) (0.0015 mmoles, 9) was dried by repeated addition and evaporation of dry DMF and then dissolved in a mixture of dry DMF (800 µL) and dry DMSO (80 µL). To this solution, imidazole (5mg, 0.08 mmole), triphenylphosphine (8 mg, 0.03 mmoles) and 2,2'-dipyridyl disulfide (5 mg, 0.02 mmoles) were added. After 1.5 h at ambient temperature, the sodium salt of the imidazolidate was precipitated by pouring the reaction mixture into 10 mL of a 1% solution of sodium iodide in acetone. The resulting precipitate was centrifuged, washed with dry acetone (3x5 mL) and then dried in vacuo for 2h. Tri-n-butylammonium pyrophosphate (200 µl of a 0.5 M soltuion on DMF) was added to the dried imidazolidate and the resulting solution was left at room temperature for 15 h. After dilution of the reaction mixture with water (3 mL), it was applied to a DEAE-Sephadex A25 column (1x20cm). Elution was with a linear gradient of triethylammonium bicarbonate (0.2-0.6 M, pH 7.5, 250/250 mLs/reservoir). Appropriate fractions were combined and evaporated to dryness in vacuo <30°. Addition and evaporation of water was necessary to remove excess triethylammonium bicarbonate buffer. The yield of product was 50 $O.D._{260}$ units or 71% overall.

The triphosphate, 10, was homogeneous with a retention time of 15.48 min on a Waters µBondapack C_{18} column (3.8 mm x 30 cm): conditions 0-50% B in A in 30 min where A=50 mM ammonium phosphate PH7 and

B=CH_3OH/H_2O(1:1). Upon extended digestion with 0.5N KOH, 10 gave two products, p5'(3'dA)2'p5'A2'(3')p and 3'-deoxyadenosine in a ratio of 1.09 to 1.00, respectively. Digestion with snake venom phosphodiesterase also gave two products identified as p5'(3'dA) and 5'AMP (ratio 2.04:1.00, respectively). Finally, treatment of 10 with alkaline phosphatase gave only a single product, tenatively identified as the "core" (3'dA)2'p5'A2'p5'(3'dA).

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